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Dual R-Smads interplay in the regulation of vertebrate neurogenesis

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During neurogenesis, multiple regulatory networks integrate extracellular and intracellular signals to ensure proper final numbers for each neuronal subtype at the end of the developmental process. The Activin/TGF-β signaling cascade is one of the main players of neurogenesis in vertebrates, balancing proliferation and differentiation of neural stem cells by regulating gene expression via the R-Smads transcription factors. Despite their equivalent upstream activation mechanism, Smad2 and Smad3 functions can be redundant or opposite depending on the particular context of the cell. We demonstrate that R-Smad simultaneously cooperate and antagonize in the regulation of gene expression in the context of vertebrate neurogenesis. We propose a model where synergism and antagonism appear as a consequence of the competition between Smad2 and Smad3 to form the different transcriptionally active heterotrimers with Smad4. This dual interplay of R-Smads significantly modulates the role of TGF-β pathway in the balance between proliferation and differentiation in the developing vertebrate spinal cord.

Keywords: neural tube, differentiation, networks, systems biology

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Transforming growth factor β (TGF-β) superfamily is one of the main classes of multi-tasking secreted proteins that drives animal development.[1] There are 2 branches in this family: bone morphogenic proteins (BMP) and Nodal/Activin/TGF-β, which orchestrate several key processes during embryogenesis.[2] Acting through Smad transcription factors, they regulate a variety of cellular functions including cell proliferation, growth arrest, differentiation and migration. The canonical TGF-β pathway begins with the binding of TGF-β ligands to different isoforms of type II and I Ser/Thr kinase receptors at the cell membrane.[3] The ligand promotes receptor dimerization, enabling the phosphorylation and activation of the receptor type I by the type II. Specifically, activated Nodal/Activin/TGF-β receptor complexes recruit and phosphorylate Smad2 and Smad3 proteins, which oligomerize with Smad4 to form transcriptionally active heterotrimers. These active complexes translocate into the nucleus where they regulate gene expression (**Fig. 1A**).[4-6]

Despite this apparently simple mechanism of activation, the Nodal/Activin/TGF-β pathway plays major and diverse roles during embryo development. The concentration gradient of Nodal is essential for the establishment of the anterior–posterior (A/P) axis and the induction of the mesendoderm layer. Subsequently, together with BMPs, they organize the left–right (L/R) asymmetry, finishing the specification of the body plan.[1,7] Counteracting BMP and Shh gradients regulate closure and dorsal–ventral (D/V) polarization of the neural tube.[8-10] BMP/Activin ligands are expressed dorsally in the newly originated neural tube,[11] whereas TGF-β 2, 3 isoforms are preferentially expressed in the notochord and the floor plate,[12,13] concomitant with Shh expression. The opposing gradients of these ligands are involved in the specification pattern for each neuronal subtype in the neural tube. Nonetheless, the particular functions of each member of the Activin/TGF-β subfamily

during neurogenesis are beginning to be understood.

In the dorsal pole of the neural tube, Activin signaling promotes the formation of the dorsal interneuron 3 (dI3) precursor cells.[14] Besides, TGF-β factors contribute to regulate the balance between proliferation and differentiation of neural stem cells in conjunction with other pathways. TGF-β counterbalances the proliferative signals of IGF1/AKT in the forebrain and Wnt/FGF8 in the midbrain.[15,16] In addition, TGF-β1 signaling participates in neuronal differentiation by modulating the expression of regulators of the cell cycle,[17,18] whereas the isoforms TGF-β2,3 are essential for the specification of dopaminergic neurons induced by Shh.[19]

The role of Activin/TGF-β signaling in the regulation of neurogenesis is ultimately mediated by the downstream transcription factors Smad2 and Smad3. Particularly, Smad3 triggers the differentiation program of ventral interneuron progenitors through transcription of bHLH proteins such as NeuroM and Mash1, inhibition of Id1,2,[20,21] and promoting cell-cycle exit by transcribing cell-cycle inhibitors p15, p21, or p27.[17,20,21] In contrast, Smad2 activity is correlated with Nodal signaling throughout embryogenesis, including A/P patterning and endoderm formation, although its specific function in vertebrate neurogenesis remains unclear.[7] However, recent studies suggest a role of Smad2 in the regulation of axonal morphogenesis in telencephalon and metencephalon,[22,23] which correlates with the pro-survival effects that TGF-β has in neurons in other contexts.[24,25] The role of Smad2 in neurogenesis appears to be related to SnoN, a modulator of axonal development and growth.[26] The SnoN pathway is receiving increased importance during embryogenesis, since recent findings suggest a high convergence with other important pathways, including Nodal/TGF-β[27] and cell cycle regulators such as p53 and APC/Cdh1.[28] Furthermore, in some cell lines, SnoN expression seems to be positively and negatively regulated by both R-Smads, revealing a potential implication of both R-Smads in this pathway.[29]

Related to the expression profile, Smad2 is widely distributed in the neuroepithelium, and latterly in the telencephalon and metencephalon.[30] Nonetheless, in the context of the developing neural tube, Smad2 was not detected in previous studies.[20] We showed that Smad2 is expressed in the ventricular zone of the developing spinal cord in the chick neural tube, and that its expression partially overlaps with the expression pattern of Smad3, which is a requirement for a potential interplay between both R-Smads at the cellular level.[31]

As reviewed above, Smad2 and Smad3 are functionally nonequivalent,[5,32] despite their common upstream activation mechanism and their 92% identity in their amino acid sequences.[32-34] Knockout (KO) studies in mice, show early embryonic lethality in Smad2 KO. This was attributed to disruption of the A/P axis within the epiblast and failure in the formation of the ectoderm, mesoderm, and endoderm.[34] In contrast, the KO of Smad3 is not embryonic lethal, although mice present deficiencies in the immune response and high predisposition to tumorgenesis.[35] In addition, Smad2 and Smad3 have been shown to recruit different co-factors and target different regulatory sequences.[36] In particular, Smad3 binds directly to a CAGA-box in the promoter of PAI-1 or c-Jun, whereas Smad2 binds indirectly through Smad4 and co-factors, such as FoxH1 and Mix families, to Activin response elements (ARE) or other regulatory sequences.[33,37] Moreover, recent studies suggest that cell-type specific master transcription factors such as Oct4, Myod1, or PU.1 determine Smad2 and Smad3 targets, and hence direct TGF-β effects in a context-dependent manner.[38]

Despite the important and multiple functions of the TGF-β signaling in many cellular processes and diseases,[39,40] there are still relevant open questions regarding to the global mechanisms that govern TGF-β dynamics and function, with the 2 R-Smads playing redundant roles in some scenarios[9,10] while antagonizing in others.[41,42]

To answer the question of how these 2 highly similar molecules targeting different DNA regulatory sequences can synergize or antagonize depending on the cellular context, we compared the function of both R-Smads in the context of vertebrate neurogenesis.[31] Our studies revealed a simultaneous cooperation and antagonism between Smad2 and Smad3 occurring at the same cellular context. Experiments performed in the chick neural tube by in ovo electroporation showed an increase of Smad3-sepecific transcription targets after Smad2-Smad3 gain of function (GOF) compared with wild-type (WT) or Smad3 GOF alone. Surprisingly, Smad2 loss of function (LOF) also induced a strong increase of Smad3 activity (**Fig. 1B**). Additionally, Smad2-Smad3 GOF resulted in an increase of the amount of neural progenitors undergoing differentiation, specifically of ventral interneurons, compared with WT or GOF of Smad3.[20] Correspondingly, LOF of Smad2 also induced a strong increase in differentiation, compared with the WT situation.

In order to address the potential underlying mechanisms that could induce this dual interplay between the R-Smads, we followed a computational approach. Previous models of the TGF-β signaling pathway focused on the dynamics of the nucleocytoplasmic shuttling of R-Smad complexes,[43] the dynamics of signal processing, the generation of transient responses,[44] and endocytosis.[45] Most of these mathematical models assume that Smad2 and Smad3 are indistinguishable. These approaches take advantage of a detailed characterization of several potential interactions in the pathway to understand the effect of different regulatory mechanisms and crosstalks.[46]

Contrary, our modeling approach focused on the R-Smads oligomerization after phosphorylation, in order to understand the dual antagonism/cooperation observed experimentally. This minimal model successfully reproduced all experimental results when we allowed the heterotrimer Smad2-Smad3-Smad4 to bind and regulate transcription in Smad3-specific regulatory sequences. We observed by co-inmunoprecipitation that Smad2 and Smad3 physically interact in a complex with Smad4, and that the amount of Smad3 bound to Smad4 depended on the amount of Smad2 available and vice versa. The affinity of phosphorylated Smad2 to form trimers with Smad2 vs. Smad3 is unknown. Nonetheless, the model predicted stronger affinity of the R-Smads

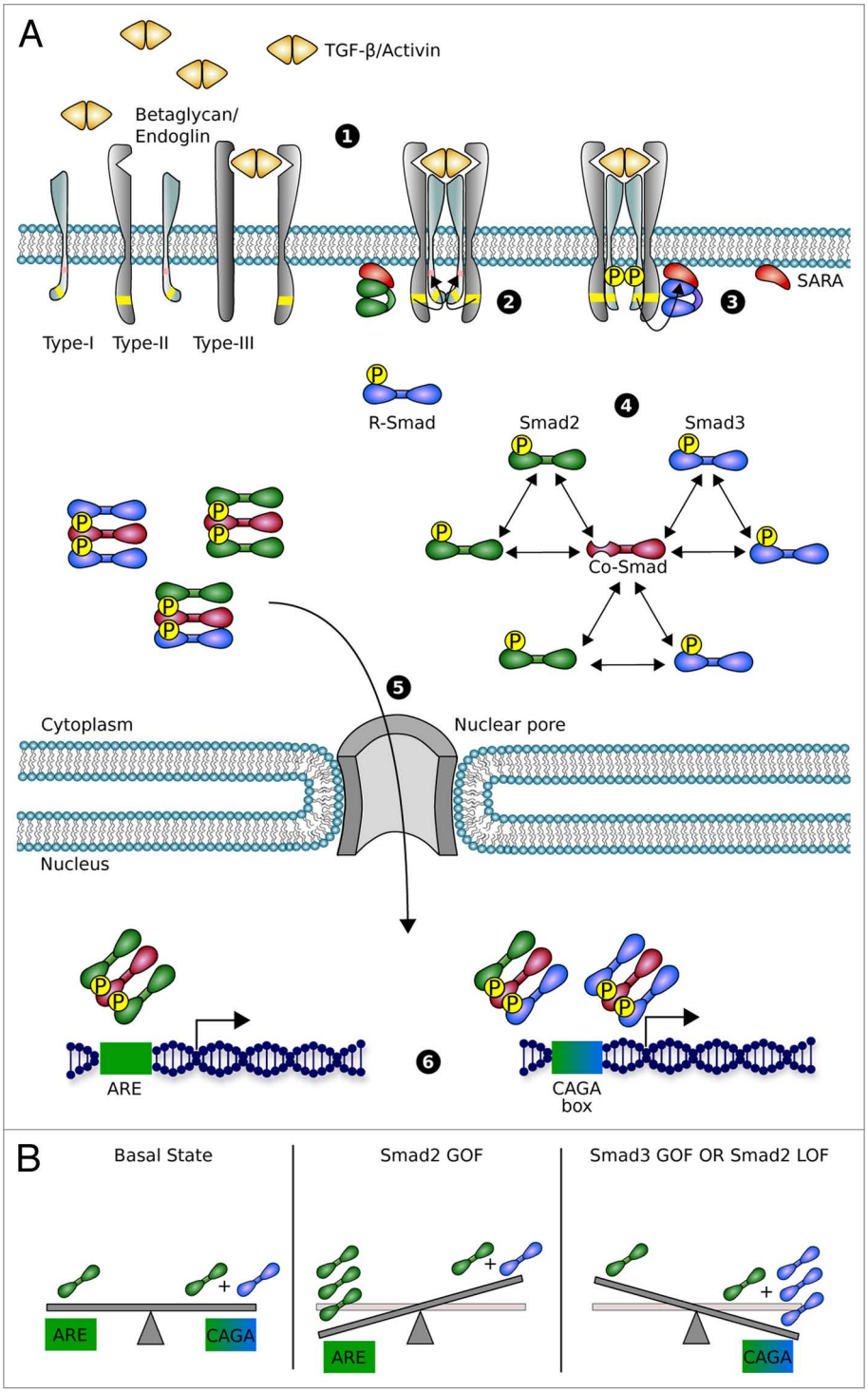

Figure 1. Scheme of the TGF-β pathway. (**A**) (1) Dimers of soluble Activin or TGF-β ligands bind their specific Ser/Thr kinase receptor type II, presented by the co-receptor betaglycan. (2) The complex oligomerizes and phosphorylates receptor type I and finally forms the tetrameric active ligand-receptor complex. (3) This complex phosphorylates R-Smad transcription factors (Smad2 in green and Smad3 in blue), recruited to plasma membrane by the anchor protein SARA. (4) Heterodimers or homodimers of R-Smad bind the Co-Smad (Smad4) to form the transcriptionally active heterotrimers. (5) Smad oligomers are actively transported to the nucleus, where (6) they bind to specific promoter regions and regulate gene expression. Specifically, Smad2-Smad2-Smad4 homotrimer is recruited to ARE while Smad3-Smad3-Smad4 homotrimer and Smad3-Smad2-Smad4 heterotrimer bind to CAGA box in the promoter regions of their target genes. (**B**) In the basal state Smad2 directed transcription (ARE) is accomplished only by Smad2-Smad2-Smad4 homotrimer while Smad3 targets (CAGA box) are regulated by both Smad3-Smad2-Smad4 and Smad3-Smad3-Smad4 trimers. Increasing the levels of Smad2 (Smad2 GOF) shifts the balance to overexpress Smad2 targets (ARE). Similarly, Smad3 GOF leads to overexpression of Smad3 targets. Depletion of Smad2 protein (Smad2 LOF) contributes not only to downregulate Smad2 targets, but also to increase Smad3 driven transcription.

to form the heterotrimer Smad2-Smad3-Smad4, than Smad2-Smad2-Smad4 or Smad3-Smad3-Smad4.

Overall, our results evidence a scenario of competition between R-Smad to form the 3 potential heterotrimers that depends on the concentration and the binding affinities of the R-Smads. Thus, the selection between cooperation and antagonism of the R-Smads is not cell type-dependent, occurring in the same cellular context as a consequence of the interaction between Smad2, Smad3, and Smad4.

Many aspects of the TGF-β pathway were not included in the analysis for the sake of simplicity. In our model, we assumed Michaelis–Menten kinetics for the phosphorylation dynamics of the R-Smads, and therefore the amount of phosphorylated R-Smads depended directly on the amount of available R-Smads after receptor activation. Moreover, we considered that protein production and degradation are balanced. Consequently, the concentration of each R-Smad was assumed constant. A more detailed analysis of our model, including a full mathematical description of the known interactions of the TGF-β pathway, remains to be studied. Potential candidates to include in the model are compartmentalization, nucleo-cytoplasmatic translocation of the R-Smads,[47] and inhibition by I-Smads (Smad6 and Smad7) and E3 ligases.[48] The I-Smads induce a well-characterized negative feedback loop that can induce nonlinear effects, such as desensitization of the pathway and robustness against perturbations.[49] Interestingly, some E3 ligases such as Arkadia have been demonstrated to positively contribute to TGF-β signaling. Arkadia has an essential role during embryogenesis because, under TGF-β activation, it degrades negative feedback inhibitors of the pathway, such as Smad7 and SnoN, a negative co-factor for Smad3/4 directed transcription.[44,50] All these potential interactions will result in a more realistic mathematical approximation to the true dynamics of the pathway, but the increasing complexity and the amount of unknown reaction parameters will complicate the analysis and the understanding of the consequences of the interplay between the R-Smads.

The arising characteristics of the TGF-β system constitute a paradigm of how the network of interactions can determine the strength, dynamics and even the function of proteins in a given pathway. These emerging characteristics induced by a complex network have been studied widely in biological systems.[51] In the context of neurogenesis, Shh graded responses have been proposed to be interpreted via a nonlinear network of interacting genes.[8] In addition, domain specification of the neural tube has been also shown to be regulated by a network of interacting genes with multiple interconnected positive and negative feedback loops.[52] For instance, a feedback in Delta-Notch signaling that mediates lateral inhibition is key in the regulation of pro-neural genes and neural differentiation.[53] In fact, the characterization of the interactions within the network has been shown to be decisive for a full understanding of different developmental processes like somite formation,[54] palate,[55] and digit formation in mice[56] and pigmentation pattern in animal skin[57] and also in treatment and prognosis of pathologies such as cancer.[58-60] These studies evidence that a complete understanding of the TGF-β and other signaling networks involved in neurogenesis should take into account not only the study of the biochemical aspects of the proteins, but also the analysis of the interaction network.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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